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APPENDIX A

Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*

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ABSTRACT Tuberculosis, caused by the intracellular pathogen *Mycobacterium tuberculosis*, is the world's leading cause of death in humans from a single infectious agent. A safe and effective vaccine against this scourge is urgently needed. This study demonstrates that immunization with the 30-kDa major secretory protein, alone or in combination with other abundant extracellular proteins of *M. tuberculosis*, induces strong cell-mediated immune responses and substantial protective immunity against aerosol challenge with virulent *M. tuberculosis* bacilli in the highly susceptible guinea pig model of pulmonary tuberculosis. Protection is manifested by decreased clinical illness including decreased weight loss, reduced mortality, and decreased growth of *M. tuberculosis* in the lungs and spleens of immunized animals compared with sham-immunized controls. This study demonstrates that purified major extracellular proteins of *M. tuberculosis* are candidate components of a subunit vaccine against tuberculosis and provides compelling support for the concept that extracellular proteins of intracellular pathogens are key immuno-protective molecules.

Tuberculosis is one of the world's most important infectious diseases. The principal etiologic agent, *Mycobacterium tuberculosis*, currently infects 2 billion people worldwide and causes 8 million new cases of active tuberculosis and 2.9 million deaths annually (1). The emergence of multidrug-resistant strains of *M. tuberculosis* is an ominous new threat to the public health.

An effective and safe vaccine against tuberculosis is sorely needed. The only currently available vaccine, bacillus Calmette–Guérin (BCG), a strain of *Mycobacterium bovis*, is of questionable efficacy (2). A live organism, BCG can cause serious and even fatal disseminated disease in immunocompromised patients, including AIDS patients (3). Another disadvantage of BCG administration is that it can cause the tuberculin skin test to become reactive, diminishing the usefulness of this valuable diagnostic test for *M. tuberculosis* infection. Largely for these reasons, BCG is not used in the United States or in several other nations (2).

A subunit vaccine, consisting of a few key molecules of *M. tuberculosis* that are capable of inducing protective immunity, could have substantial advantages over BCG or other whole-bacterium vaccines. Since the subunit vaccine would consist of only a few selected molecules (e.g., proteins) rather than the thousands of molecules of different types (proteins, lipids, glycolipids, lipopolysaccharides, nucleic acids, etc.) that make up a whole bacterium, it is more likely to be safe. Since the subunit vaccine can be constructed so as to eliminate irrelevant or even immunosuppressive components of the whole bacterium, it might induce a stronger protective immune response than a whole-bacterium vaccine. Finally, in contrast to BCG, the subunit vaccine can be rigorously standardized, allowing the production and use of a consistent preparation and hence meaningful predictions regarding its efficacy and safety.

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The design of a subunit vaccine against *M. tuberculosis* must take into account certain features of this pathogen that distinguish it from most others against which successful vaccines have been developed. First, and most importantly, *M. tuberculosis* is an intracellular pathogen that survives and multiplies within mononuclear phagocytes. It is phagocytized via complement and mannose receptors on the host cell surface (4, 5). Thereafter, the organism resides intracellularly in a membrane-bound phagosome that has class II major histocompatibility complex (MHC) molecules and endosomal characteristics, including transferrin receptors (6), but does not fuse with lysosomes (6, 7). Second, cell-mediated immunity, as opposed to humoral immunity, plays a dominant role in host defense against *M. tuberculosis* and other intracellular pathogens.

Intracellular pathogens, including *M. tuberculosis* and the intracellular bacterial parasite *Legionella pneumophila*, secrete or otherwise release proteins into their phagosomes within human mononuclear phagocytes (refs. 8 and 9; D. L. Clemens and M.A.H., unpublished data). These facultative intracellular pathogens also release these proteins, collectively referred to as extracellular proteins, into their extracellular milieu when growing in broth culture; from such cultures, the proteins are readily purified.

Horwitz and coworkers (10–14) have proposed three hypotheses regarding the role of extracellular proteins of intracellular pathogens in protective immunity and the potential for their use in a subunit vaccine. (i) Extracellular proteins should play a key role in inducing cell-mediated immune responses that provide immunoprotection against these pathogens during natural infection. Such proteins, by virtue of their release by live organisms into their intracellular compartment in the host cell, are available for proteolytic processing and subsequent presentation on the surface of the infected host cell as MHC-bound peptide fragments. These surface-exposed fragments would allow the host immune system to recognize live pathogens sequestered within a host cell and to exert an antimicrobial effect against them. In particular, T cells may activate the host macrophage, allowing it to inhibit multiplication of intracellular organisms, or they may lyse the host cell, thereby denying the pathogens an intracellular milieu in which to multiply. In support of this hypothesis is the finding that guinea pigs infected with *L. pneumophila* or *M. tuberculosis* develop a strong cell-mediated immune response against immunoprotective extracellular proteins of these organisms (10–15).

(ii) Immunization of a naive host with extracellular proteins of intracellular pathogens—particularly in the case of pathogens such as *L. pneumophila* and *M. tuberculosis*, which reside within a phagosome rather than free in the cytoplasm in host cells—would induce a population of lymphocytes capable of later recognizing and exerting an immune response against infected host cells. These lymphocytes would recognize in-

Abbreviations: BCG, bacillus Calmette–Guérin; cfu, colony-forming unit(s); DTH, delayed-type hypersensitivity; MHC, major histocompatibility complex; SAF, Syntex adjuvant formulation.

fected host cells by identifying MHC-bound fragments of extracellular proteins displayed on the host cell surface consequent to the release of the proteins by the intracellular pathogen. A necessary corollary is that the extracellular proteins are processed and presented similarly in a given host whether they are exogenously delivered to antigen-presenting cells via vaccination or released by intracellular pathogens into phagosomes of infected host macrophages. In support of this hypothesis, immunization of naive hosts with purified extracellular proteins of *L. pneumophila* induces strong protective immunity to a lethal aerosolized dose of *L. pneumophila* in the guinea pig model of Legionnaires' disease (11–13). Moreover, immunization of naive hosts with a crude extract of extracellular proteins of *M. tuberculosis* induces protective immunity to challenge with aerosolized highly virulent *M. tuberculosis*, Erdman strain, in the guinea pig model of pulmonary tuberculosis (14). This observation was recently confirmed in mice parenterally challenged with *M. tuberculosis* (16).

(iii) Among the extracellular proteins of intracellular pathogens, the ones released in greatest abundance will be among the most effective in inducing immunoprotection. Such proteins, by virtue of their abundance in the phagosome, would be processed and presented most frequently, and, all other things being equal, therefore induce a particularly strong cell-mediated immune response. In support of this hypothesis, immunization with either of the two most abundant extracellular proteins of *L. pneumophila* induces strong immunoprotection (11–13). Now we have examined whether this hypothesis can be extended to major extracellular proteins of *M. tuberculosis*.

MATERIALS AND METHODS

Bacteria. *M. tuberculosis* Erdman strain (ATCC 35801) was cultured on 7H11 agar (14), subjected to gentle sonication to obtain a single-cell suspension, and frozen at –70°C for use in animal challenge experiments.

Protein Purification. The 32-, 30-, 24-, 23-, and 16-kDa proteins were purified from cultures of *M. tuberculosis* grown in 7H9 broth (pH 6.7) prepared with glycerol but without albumin and Tween. The bacteria were cultured at 37°C in 5% CO₂ for 2½ weeks from an initial OD₅₄₀ of 0.05 to a final OD₅₄₀ of 0.5. The culture supernatant was harvested by filtration, concentrated from 10 liters to 100 ml by membrane filtration, and sequentially brought to 10%, 60%, and 95% saturation with ammonium sulfate and left to stand overnight. The 32-, 30-, 24-, and 16-kDa proteins precipitated at 10–60% and the 23-kDa protein at 60–95%. The proteins were purified further by chromatography on DEAE-Sepharose CL-6B. The 16-, 32-, 30-, and 24-kDa proteins were eluted at 50, 70, 140, and 250 mM NaCl, respectively, in 25 mM Tris Cl (pH 8.7) and the 23-kDa protein was eluted at 130 mM NaCl in 50 mM Bistris Cl (pH 7.0). Finally, the five proteins were purified to homogeneity by gel filtration on Superdex 75.

The 71-kDa protein was purified separately from a culture of *M. tuberculosis* grown in 7H9 medium (pH 7.5) in 0% CO₂ and heat shocked at 42°C for 3 hr weekly. The protein, which precipitated at 40–95% saturation with ammonium sulfate, was purified by chromatography on ATP-agarose, essentially as described (17).

Immunization of Animals with Purified Proteins. Specific-pathogen-free outbred male Hartley strain guinea pigs (250–300 g) (Charles River Breeding Laboratories) in groups of six were immunized intradermally with purified extracellular proteins of *M. tuberculosis* in Syntex adjuvant formulation (SAF) consisting of Pluronic L121, squalane, and Tween 80, and, in the first immunization, alanyl muramyl dipeptide (18). In experiments testing cutaneous delayed-type hypersensitivity (DTH) to individual proteins, animals were immunized two or three times, 3 weeks apart, with 100 µg of each protein. In

challenge experiments, animals were immunized three or four times with combinations of proteins containing 2, 20, or 100 µg of each protein in the combination. Control animals were sham-immunized with adjuvant only.

Cutaneous DTH to Purified Proteins. Three weeks after the last immunization, guinea pigs were shaved over the back and injected intradermally with 10, 1, and 0.1 µg of the immunogen. Animals immunized with combinations of proteins were tested with the whole combination (10 µg of each protein) and separately with each individual protein in the combination. After 24 hr, the diameter of erythema and induration was measured.

Protective Immunity to Aerosol Challenge. Three weeks after the last immunization and immediately after skin testing, animals were challenged with an aerosol generated from a 10-ml single-cell suspension containing 5 × 10⁵ colony-forming units (cfu) of *M. tuberculosis* (14). This aerosol dose delivered ≈200 live bacilli to the lungs of each animal. Afterwards, guinea pigs were housed in stainless steel cages within a laminar-flow biohazard safety enclosure and allowed free access to standard laboratory chow and water. The animals were observed for illness and weighed weekly for 9 or 10 weeks and then killed. The right lung and spleen of each animal was removed and cultured for cfu of *M. tuberculosis* (14).

RESULTS

Analyses of Major Extracellular Proteins Studied. To examine whether purified major extracellular proteins of *M. tuberculosis* are immunoprotective, we purified the 12 most abundant proteins of the ≈100 extracellular proteins in *M. tuberculosis* culture filtrates, and on the basis of preliminary data, selected 6 for further study. We then purified >25 mg of each of the 6 proteins to homogeneity from ≈150 liters of culture filtrates of *M. tuberculosis* grown for 2½ weeks. Under denaturing conditions, these proteins had apparent masses of 16, 23, 24, 30, 32, and 71 kDa. Each protein yielded a single band on SDS/PAGE and a single N-terminal amino acid sequence (Fig. 1; Table 1). The 30-kDa protein, the major secretory protein of *M. tuberculosis*, accounted for almost a quarter of the total extracellular protein in the culture filtrate.

A search of protein sequence databases for the N-terminal sequences of the six proteins revealed high homologies with

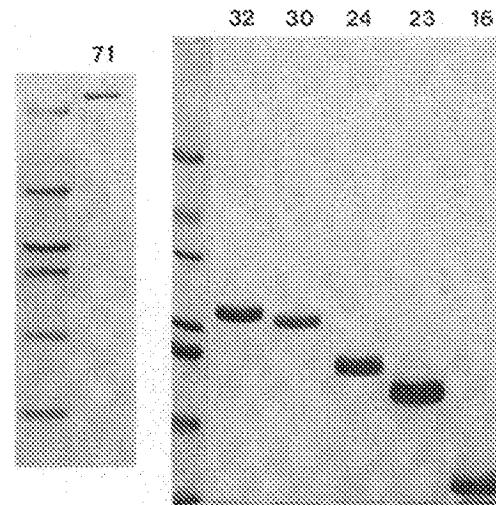


FIG. 1. SDS/PAGE analyses of six major extracellular proteins of *M. tuberculosis* studied in this report. After electrophoresis, proteins were stained with Coomassie brilliant blue R. The apparent subunit mass (kDa) of each protein is marked above the lane containing it. Standards, in the left lane of each set, are 66, 45, 36, 29, 24, 20.1, and 14.2 kDa.

previously described proteins of *M. tuberculosis* and/or *M. bovis*. The N-terminal sequences of the 30-, 32-, 23-, and 71-kDa proteins, previously designated antigen 85B, antigen 85A, superoxide dismutase, and hsp71 (DnaK analog), respectively, were 100% identical to the sequences predicted from the respective genes of *M. tuberculosis* (refs. 19 and 20; R. Young and D. Young, personal communication) or *M. bovis* (21). The N-terminal sequences of the 24- and 16-kDa proteins were 90% identical to the N-terminal 20 aa of a protein MPT51 (22) and 100% identical to the N-terminal 5 aa of protein MPT63 (22), respectively.

Cutaneous DTH to Purified Major Extracellular Proteins. We initially investigated whether immunization of guinea pigs with each of the purified extracellular proteins would induce a cell-mediated immune response. Immunized guinea pigs consistently exhibited a 3-fold or greater area of erythema and induration than sham-immunized (SAF only) control animals to each of the proteins. The level of the cutaneous responses was skin-test dose-dependent ($10 > 1 > 0.1 \mu\text{g}$).

Protective Efficacy of Major Extracellular Proteins. In preliminary experiments, only the 30-kDa protein, the major secretory protein, consistently induced protection by itself; the 71-kDa protein by itself induced protection in two of four experiments. We therefore examined the immunoprotective capacity of the 30-kDa protein alone or in combination with other proteins in the four experiments described in detail below (Exps. A–D in Figs. 2 and 3). Combinations of proteins were used because of their potential to induce a cell-mediated immune response to a broader range of protein epitopes and hence stronger protective immunity than a single protein. Two combinations were studied. Combination I consisted of 30-, 32-, 16-, 23-, and 71-kDa proteins. Combination II consisted of 30-, 32-, 16-, 23-, and 24-kDa proteins.

In each of the four experiments, we immunized the animals in groups of six with the purified proteins in SAF three or four times and then skin-tested the immunized and sham-immunized control animals with the immunogen just before aerosol challenge with *M. tuberculosis*. In all cases, the immunized animals exhibited strong cutaneous DTH responses to each of the immunogens that were markedly greater than those of controls (Fig. 2). Animals immunized with a combination of proteins were also skin-tested with the individual proteins of the combination and uniformly exhibited cutaneous DTH responses to each of the individual proteins which were significantly greater than those of controls.

We then challenged immunized and control animals with a large aerosol dose of the highly virulent *M. tuberculosis* Erdman strain. The airborne route of infection is the natural route

Table 1. N-terminal sequence and relative abundance of six major extracellular proteins

Mass, kDa*	N-terminal amino acid sequence				% of total†	Rank‡
	5	10	15	20		
30	FSRPG	LPVEY	LQVPS	PSMGR§	22	1
32	FSRPG	LPVEY	LQVPS	PSMGR§	15	2
16	AYPIT	GKLGS	ELTMT	DTVGQ	11	3
23	AETYI	PDLDW	DYGAL	EPHIS	7	5
24	APYEN	LMVPS	PSMGR	DIPVA	7	6
71	ARAVG	IDLGT	TNSVV	SVLEG	4	10

*Based on SDS/PAGE analysis.

†Estimated percent of total extracellular proteins of $>10 \text{ kDa}$. For the 71-kDa protein only, the percentage shown is for a heat-shocked culture.

‡Order of abundance.

§Extended N-terminal sequence of 30- and 32-kDa proteins (dashes indicate identity):

	25	30	35	40
30	DIKVQ	FQS GG	NNSPA	VYLLD
32	-----	-----	A-----	L-----

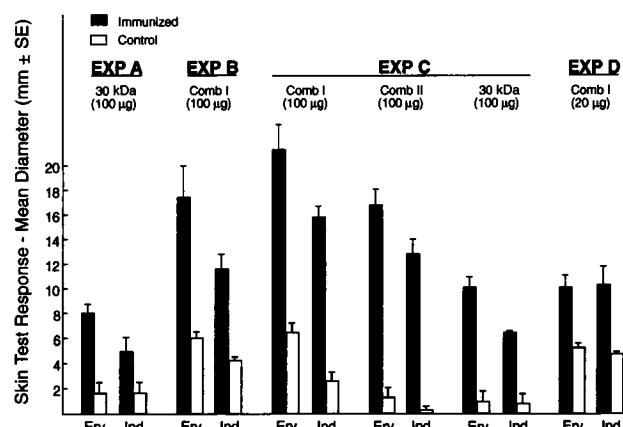


FIG. 2. Immunization with combinations of major extracellular proteins induces strong cutaneous DTH to the immunizing agent. In four experiments (A–D), guinea pigs in groups of six were immunized three times (A and B) or four times (C and D) with purified major extracellular proteins of *M. tuberculosis* in SAF adjuvant at the dose indicated. Three weeks later, the animals were skin-tested with an intradermal injection of $10 \mu\text{g}$ of the immunizing agent and the extent of erythema (Ery) and induration (Ind) was measured after 24 hr.

of infection for pulmonary tuberculosis. A large dose was used to induce measurable clinical illness in 100% of control animals within a relatively short time (2–3 months). Control animals used for skin testing (Fig. 2) were not challenged. Instead, a separate group of control animals that had not been skin-tested was challenged to eliminate the possibility that the skin test itself might alter the outcome.

Immunization with purified extracellular proteins protected challenged animals from clinical illness, as evidenced by impressive protection against weight loss (Fig. 3), a major physical sign of tuberculosis in humans, and a hallmark of tuberculosis in the guinea pig model (14). In comparison with animals immunized with the purified proteins, control animals lost 11–25% of their total body weight (Table 2). Differences in weight gain (or loss) between immunized and control animals were highly significant (Table 2). Both immunized and control animals lost weight during weeks 3 and 4 after challenge (Fig. 3). Thereafter, immunized animals but not controls consistently regained the lost weight. In fact, animals immunized with combination I ($100 \mu\text{g}$) or the 30-kDa protein gained weight from week 4 after challenge to the end of the experiment at a rate comparable to that of uninfected control animals [$15.0 \pm 2.5\%$ (mean \pm SE) weight gain for immunized animals vs. $16.4 \pm 1.0\%$ weight gain for uninfected controls in the same experiments]. However, the rate of weight gain during this time period for animals immunized with combination II was less than that of uninfected controls (8.2% vs. 15.4%) in the one experiment in which this combination was tested.

Immunization with purified extracellular proteins also protected challenged animals from death. The guinea pig model of pulmonary tuberculosis is primarily a chronic disease model as opposed to a mortality model during the first few months after challenge with *M. tuberculosis*; however, in two experiments (A and B), more than one animal in any group died during the observation period. Immunized animals had much lower mortality than controls (33% vs. 83%, in Exp. A and 0% vs. 50%, in Exp. B).

Immunization with purified extracellular proteins protected animals against growth of *M. tuberculosis* in the lungs and spleen. At the end of the observation period in Exps. A–D, surviving guinea pigs were killed and the right lung and spleen of each animal was removed aseptically and assayed for cfu of *M. tuberculosis*. Guinea pigs immunized with either the 30-kDa

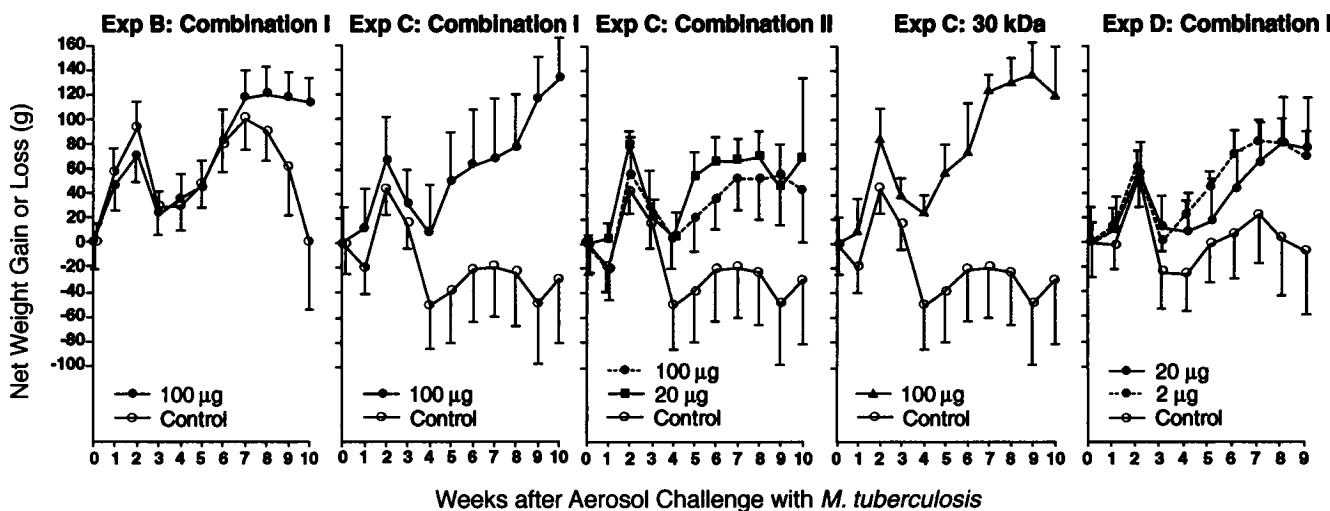


FIG. 3. Guinea pigs immunized with purified *M. tuberculosis* extracellular proteins are protected against weight loss after challenge with *M. tuberculosis*. After skin testing, the animals described in Fig. 2 were challenged with *M. tuberculosis* and weighed weekly for 9 or 10 weeks. Data are the mean net weight gain or loss \pm SE for each group of animals compared with their weight at the start of the challenge. Exp. A is not included because the high mortality (83%) of the control group precluded a meaningful analysis of weight differences between immunized and control animals.

protein (Exps. A and C) or combination I (100 µg) (Exps. B and C) had $\log \text{cfu} = 6.8 \pm 0.2$ (mean \pm SE) in their right lungs vs. 7.6 ± 0.4 in the right lungs of controls. Within the same experiment, immunized animals had 0.7 ± 0.1 log unit fewer cfu in their right lungs (mean of the differences \pm SE) than controls. In the same experiments, immunized guinea pigs had $\log \text{cfu} 6.0 \pm 0.3$ in their spleens vs. 6.8 ± 0.6 in the spleens of controls. Within the same experiment, immunized animals had 0.6 ± 0.3 log unit fewer cfu in their spleens than controls.

Immunization with purified extracellular proteins also protected challenged animals against lung destruction. On gross inspection, lungs of immunized animals had fewer and smaller lesions than controls (Fig. 4). Lesions in control animals were frequently so large and coalescent that they appeared to occupy almost the entire lung. This made quantitation difficult. However, differences in the extent of lung pathology between immunized and control animals were readily apparent to an observer blinded to the identity of each group.

Table 2. Protection against weight loss by immunization with purified extracellular proteins in guinea pigs challenged with *M. tuberculosis*

Exp.	Immunization group*	Dose, µg	% protection		
			% weight change†	from weight loss‡	P§
B	Sham	—	0		
	Combination I	100	19	19	1×10^{-7}
C	Sham	—	-4.2		
	Combination I	100	20.9	25	5×10^{-5}
	Combination II	100	6.7	11	2×10^{-3}
	Combination II	20	11.3	16	2×10^{-3}
D	30 kDa	100	19.4	24	1×10^{-6}
	Sham	—	-1.1		
	Combination I	20	11.2	12	0.02
D	Combination I	2	9.6	11	0.04

*Combination I consisted of 30-, 32-, 16-, 23-, and 71-kDa proteins; combination II consisted of 30-, 32-, 16-, 23-, and 24-kDa proteins.

†[(mean weight at end of challenge) - (mean weight at start of challenge)]/mean weight at start of challenge $\times 100$.

‡(Mean % weight gain of immunized animals) - [mean % weight gain (loss) of controls].

§P value by analysis of variance with repeated measures for differences in absolute weight gain (loss) between immunized and control animals.

DISCUSSION

This study demonstrates that immunization with purified extracellular proteins of *M. tuberculosis* induces protective immunity against *M. tuberculosis* in the guinea pig model of pulmonary tuberculosis. Immunized animals are substantially protected against weight loss, death, and growth of *M. tuberculosis* in their lungs and spleens. Together with previous studies demonstrating the immunoprotective capacity of purified extracellular proteins of *L. pneumophila* (11–13), this study supports the concept that extracellular proteins of intracellular parasites are key immunoprotective molecules and candidates for inclusion in subunit vaccines.

The guinea pig model is especially relevant to human tuberculosis clinically, immunologically, and pathologically. In contrast to the mouse and rat, but like the human, the guinea pig (i) is susceptible to low doses of aerosolized *M. tuberculosis*; (ii) exhibits strong cutaneous DTH to tuberculin; and (iii)

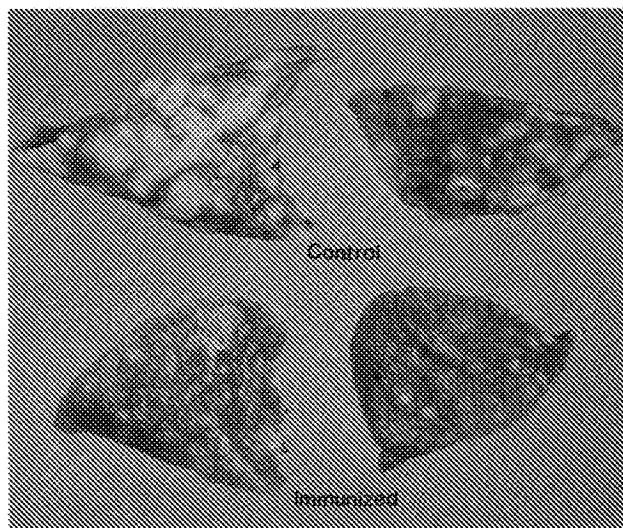


FIG. 4. Protection against lung destruction by immunization with purified *M. tuberculosis* proteins. This representative photograph depicts the two lobes of the left lung of a control animal (Upper) and of an animal immunized with combination I proteins (Lower) in Exp. C. The lung of the control animal has large lesions that have coalesced. The lung of the immunized animal has pinpoint lesions.

displays Langhans giant cells and caseation in pulmonary lesions (23). However, whereas only about 10% of immunocompetent humans who are infected with *M. tuberculosis* develop active disease over their lifetime (half early after exposure and half after a period of latency), infected guinea pigs always develop early active disease. While guinea pigs differ from humans in this respect, the consistency with which they develop active disease after infection with *M. tuberculosis* is an advantage in trials of vaccine efficacy.

Immunization of guinea pigs with purified major extracellular proteins of *M. tuberculosis* induced protective immunity against a large challenge dose of the organism (≈ 200 live organisms to the lungs). Although impressive, protection was nevertheless incomplete. *M. tuberculosis* multiplied by several orders of magnitude even in immunized animals, although less so than in controls. Perhaps the immunization regimen can be changed to induce even stronger protection. In any case, differences in lung cfu between immunized and control animals only slightly larger than those in this study (1–1.5 log units) were similarly associated with a major difference in clinical outcome in a study of the immunoprotective potential of the major secretory protein of *L. pneumophila* (11). The protection against *M. tuberculosis* challenge in this study, in terms of differences in lung cfu between immunized and control animals, was comparable to that afforded by adoptive transfer of immune T cells from guinea pigs or mice infected with *M. tuberculosis* to syngeneic recipients (24, 25). Although we made no attempt to choose a challenge–necropsy interval that would maximize differences in lung cfu between immunized and control animals, the level of protection was also comparable to that afforded guinea pigs and mice by immunization with live BCG vaccine in several studies (16, 26–30).

Despite its drawbacks, BCG remains the standard against which new vaccines ultimately will be compared. BCG has been shown to induce protective immunity by one measure or another in numerous animal studies. The methods employed in those studies are so diverse that our study cannot be compared with them in any meaningful way. A side-by-side comparison is required, as in the recent study that showed the induction of comparable protection in mice by immunization with BCG or a crude extract of extracellular proteins (16). BCG is known to produce at least several extracellular proteins homologous with major *M. tuberculosis* extracellular proteins. Consequently, immunization with live BCG might induce cell-mediated immune responses to the homologous *M. tuberculosis* proteins. Indeed, the immune response to these proteins may be largely responsible for the protective efficacy of BCG. Nevertheless, immunization with purified *M. tuberculosis* extracellular proteins may induce a more efficacious response to these proteins than immunization with BCG for several reasons. (i) Immunization with selected extracellular proteins would focus the immune response on a few key immunoprotective proteins rather than on the entire repertoire of mycobacterial proteins including many irrelevant to protective immunity. (ii) While the *M. bovis* extracellular proteins that have been sequenced are similar to those of *M. tuberculosis*, they are not identical and thus may lack key immunoprotective epitopes of *M. tuberculosis* proteins (iii) Mycobacteria contain a number of nonprotein molecules shown to suppress T-cell proliferation, including lipoarabinomannan, lipopolysaccharide, and arabinogalactan.

While the concept that extracellular proteins of *M. tuberculosis* are key immunoprotective molecules has yet to be extended to humans, our observation that these proteins can

induce protective immunity against tuberculosis in the highly susceptible guinea pig gives reason for optimism that these proteins can induce immunoprotection in humans, who have much greater innate resistance to the development of active tuberculosis.

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